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Bassoon and Piccolo maintain synapse integrity by regulating protein ubiquitination and degradation

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 August 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees.

As you can see below, the referees find the study interesting, well executed and appropriate for the journal. They bring up some specific issues as detailed below, which I expect you will be able to address. Given the positive comments I'd like to invite you to submit a revised manuscript, taking the raised issues into account. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the concerns raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

I look forward to seeing the revised version!

REFEREE REPORTS

Referee #1

Understanding how presynaptic terminals are formed and properly maintained is central to Neuroscience. Waites et al. developed a tour-de-force approach using tricistronic vectors that were incorporated into viral particles combined with state-of-the-art confocal microscopy analysis in order to address the role of piccolo and bassoon at the presynapse. They found that an efficient

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knockdown of both proteins lead to an important decrease in the levels of different presynaptic proteins, compatible with a global degradation of presynaptic proteins. They also observed that knockdown of piccolo and bassoon lead to decreased formation of normal synaptic vesicles and to the appearance of deformed ones, which are positive for multi-vesicular bodies and endo-lysosomal markers. In addition, they identified that the zinc-finger domains of both bassoon and piccolo interact with the E3 ubiquitin-ligase siah-1, inhibiting siah-1 activity. Moreover, the authors found that knockdown of siah-1 decreased the protein degradation promoted by piccolo and bassoon knockdown, suggesting that piccolo and bassoon control the levels of presynaptic proteins through their interaction with and modulation of siah-1.

In sum, Garner group elegantly show in this manuscript that piccolo and bassoon are essential to maintain the presynapse through controlling protein degradation via siah-1. The data presented in this manuscript is very exciting and it was elegantly thought and written. The experiments were very well conducted and analyzed, and this manuscript is of great interest to readers and deserves to be published in the EMBO J. Nevertheless, the manuscript would benefit if the authors choose to improve a few points, especially those related to the ubiquitin system.

Major points:

1) Fig. 1. The data that the knockdown of piccolo and bassoon decrease the steady-state of several presynaptic proteins is very interesting and convincing. However, to characterize it as protein degradation, the authors should carry out pulse-chase or cycloheximide chase experiments to prove that indeed it is protein degradation and not changes in transcription or translation, for instance.

2) Fig. 6. The authors used lysosomal, proteasomal and E1 inhibitors to show that the protein decrease in the knockout condition is due to ubiquitination and targeting to the proteasome and lysosome. Indeed they observe a significant increase in the levels of proteins in the presence of these inhibitors when piccolo and bassoon were knocked down. However, no accumulation of proteins was observed in the control condition. Does this mean that degradation of presynaptic proteins is normally not affected by these pathways? Also, this finding is not compatible with the effect of UbK0 in increasing the protein levels also from control (Fig. 7).

3) Fig. 5. The authors observe that the abnormal vesicles that accumulate when piccolo and bassoon are knocked down are positive for lysotracker. They also observe that lysosomal inhibition by chloroquine and leupeptin are the most efficient in preventing protein degradation when piccolo and bassoon are suppressed (Fig. 6). Since lysosomal degradation of proteins may be predominantly linked to non-ubiquitinated proteins, is it possible that the lysosomal degradation in the double knockdown condition may not be related to protein ubiquitination only? Is it possible that macroautophagy may also be involved?

4) Fig. 7D. The data on the ubiquitination of presynaptic proteins could be improved. For instance, the levels of immunoprecipitated proteins should be shown for both Munc13 and VAMP2. Also, it is not clear if the detection with ubiquitin antibodies reveals polyubiquitination since VAMP2 does not appear as a smear and Munc13 had the region of high molecular weight cut. Fig. 7E should also be done again since the current one probably reflects one or very few attachments of ubiquitin rather than polyubiquitination, which should be driving the degradation of proteins based on Fig. 7A using UbK0. If the polyubiquitination is robust in the absence of piccolo and bassoon, perhaps the addition of epoxomicin with or without lysosomal inhibitors may facilitate observing the differences in ubiquitination.

5) Fig. 8. The authors mention that siah-1 was pooled out as an interactor of bassoon in a yeast-two hybrid screen. However, no details about the siah-1 region that was isolated in the screenings were provided. Since most siah substrates interact with the region C-terminus to the RING finger (sina domain), the finding that bassoon interacts with the RING finger is particularly interesting. Thus, the siah-1 yeast two-hybrid clone(s) obtained in the screening should also enclose the RING domain, which would confirm the IP data using the fragments of the protein (Fig. 8D). It is also important to mention in Figure S4A the identity of siah-1 construct used to co-transform yeast with the different parts of bassoon.

6) Even though more artificial that primary neuronal cultures, I believe that simple biochemistry

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with transfected HEK293 cells could strengthen the idea that knockdown of piccolo and bassoon activates the catalytic activity of siah-1 leading to increased ubiquitination and degradation of substrates.

Minor points:

1) The authors mention at the Results section (p. 11) that ubiquitination of one or more ubiquitin molecules target proteins for lysosomal or proteasomal degradation. Although technically correct, this information is not very precise since most proteins targeted to the proteasome require the attachment of four or more ubiquitin molecules. As for the lysosomes, even though multi-monoubiquitination or K63 polyubiquitination can promote lysosomal degradation of some proteins, it is not clear if proteins in general require ubiquitination to be degraded by this pathway. Therefore, the authors should try to rephrase their explanation.

2) No details about the yeast two-hybrid system were provided at the Materials and Methods section. This information should be added to the section.

Referee #2

In their Ms Banker and colleagues have investigated the function of the giant AZ proteins piccolo (Picc) and bassoon (Bsn) in presynaptic function using lentivirally encoded shRNA that eliminates expression of both proteins. They remarkably find an unexpected and novel role for Bsn/ Picc in maintaining presynaptic integrity, a function that at least partially depends their ability to bind to and negatively regulate the E3 ubiquitin ligase Siah1. These findings not only reveal a novel role for Bsn/ Picc in the presynapse but they also explain why previous studies using KO mice (or hypomorphs in some cases) have failed to produce strong phenotypes. Furthermore, the new work suggests that the reported effect of Bsn/ Picc on synaptic vesicle clustering reported earlier may rather have originated from enhanced presynaptic degradation as the mice used turned out to be hypomorphs.

Overall, this is an interesting study and the majority of the data are of high quality. A few points, however, ought to be addressed before publication.

1. The data regarding CHMP2 accumulation in neuronal cell bodies are less than compelling. Whether or not these antibody stainings are specific seems doubtful based on the hazy images provided. I'd rather suggest to go for other MVB markers or alternatively to make sure that the signal is specific (i.e. by comparison with KD cells). Also, does one observe transport intermediates in axons in DKD neurons that undergo retrograde traffic? If so, do these stain positive for MVB markers? This should become particularly overt if lysosomal degradation is blocked by inhibitors.

2. I am puzzled by the fact that the claimed ubiquitinated form(s) of VAMP2 in Fig. 7 runs at about 18 kDa, i.e. the MW of VAMP2 in the ABSENCE of Ub! This is odd and makes me wonder about the quantifications. Clearly, better and more conclusive evidence in favor of the accumulation or formation of Ub-conjugated presynaptic proteins is required. This is a key point in my opinion.

3. There is a poor match betwen the quantifications in Fig 1C and the blots shown in panel B. In fact, in none of the lanes do I see any evidence of presynaptic proteins loss at DIV9, although the quantifications in panel C suggest a profound loss of at least Picc and Bsn at this stage. The same concern holds true for the images in Fig. 2B: The DKD neurons shown appear to contain plenty of Picc and no difference to the scrambled control is overt.

4. I miss any kind of evidence that the ability of Siah1 to foster presynaptic degradation is related to its ability to bind to Bsn/ Picc. For example, one would predict that OE (see S5E, F) of a Bsn/ Picc binding defective mutant of Siah1 should have no effect on presynaptic protein levels similar to an enzymatically inactive version. This should be straightforward to test.

5. As rescues by Siah1 KD or OE of the Zn finger domains of Picc/ Bsn are incomplete I would suggest to discuss possible explanations for this; i.e. other degradation pathways or E3 ligases that may associate with the AZ.

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Referee #3

Waites et al: Bassoon and Piccolo maintain synapse integrity by regulating protein ubiquitination and degradation.

In this manuscript, Waites et al identify a new and surprising function for the two high molecular weight constituents of the presynaptic active zone Bassoon and Piccolo. They find that eliminating both proteins by RNAi leads to specific degradation of a wide range of synaptic proteins. They can further show that this degradation is due to ubiquitination and degradation. By a combination of pulldown experiments, knockdown and expression of interfering protein fragments they succeed to show that the E3 ubiquitin ligase Siah1 is negatively regulated by Bassoon and Piccolo and is responsible for the degradation seen in their absence.

I found the manuscript very interesting and overall convincing. The data set is extensive, and read-outs include both immunocytochemistry and electron microscopy. The experiments are very well conceived and carried out. Physiological data are not presented and would be a nice addition, but the story is complete without them. Among the most notable advances of this paper is that the authors succeed in almost completely eliminating both Bassoon and Piccolo, something which has not previously been achieved due to the difficult genetic structure of these huge proteins. Moreover, the authors demonstrate very convincingly that the degradation seen in the absence of Bassoon and Piccolo is due to Siah1, by knockdown of Siah1 and by expression of Bassoon fragments. These experiments conclusively establish the mechanistic cause of the degradation seen in the absence of Bassoon and Piccolo. These data will be extremely interesting to anyone studying synaptogenesis and synaptic remodelling. The manuscript is well written and very clear. There are a few points to settle before the paper is published:

Specific points:
1. I find the lack of consistency between the Western Blots of Fig. 1B-C and the stainings in f.instance Fig. 2 confusing. It seems that at DIV14, most tested proteins are basically gone from the Western (Fig. 1B), but in Fig. 2B, the stainings for Piccolo and Bassoon at DIV14 look normal. Why is this? The fluorescence in the immunostaining should be quantified and compared between knockdown and control construct, and the degree of reduction compared to the Western. In addition, the experiments in Fig. 2 (and most of the rest of the paper) were carried out with another viral construct (expressing EGFP-SV2 instead of EGFP) than the construct used for protein quantification in Fig. 1. Given the discrepancy between Fig. 1 and 2, the authors should make sure that there is no difference in the degree of knockdown between constructs (for instance because of a lower virus titer of the longer construct), and this should be mentioned in the paper.

2. The lack of colocalization between EGFP-SV2 and piccolo/bassoon. I am not convinced that this is a real phenomenon, or what it would mean if it were. It might be a consequence of the changes in intensity of SV2 and/or Piccolo/Basson spots, which makes it harder to detect the colocalization. If the phenomenon is real, I guess it might mean that those synapses completely devoid of Piccolo/Bassoon experience an even larger breakdown of EGFP-SV2? Or what is the author's interpretation of these results? - formation of SV2-clusters outside of synapses?

1st Revision - authors' response 16 December 2012

Referee #1:

This referee is very supportive of the study, commenting that “The data presented in this manuscript is very exciting and it was elegantly thought and written. The experiments were very well conducted and analyzed, and this manuscript is of great interest to readers and deserves to be published in the EMBO J.” The referee requested that a few additional issues be addressed prior to publication of this study. We thank the referee for these positive comments and address his/her concerns below.

1) Fig. 1. The data that the knockdown of piccolo and bassoon decrease the steady-state of several presynaptic proteins is very interesting and convincing. However, to characterize it as protein

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degradation, the authors should carry out pulse-chase or cycloheximide chase experiments to prove that indeed it is protein degradation and not changes in transcription or translation, for instance.

This is a good suggestion, and we have now performed cycloheximide-chase experiments in control and DKD neurons (shown in **Figure S3**). Each of the three proteins examined (Munc13-1, VAMP2, SNAP-25) has a shorter half-life in the Bassoon/Piccolo DKD background. These findings are consistent with data shown in Figure 6, illustrating that inhibition of lysosomes, proteasomes, or ubiquitination for ~18 hours (using the pharmacological agents chloroquine/leupeptin, epoxomicin, and ziram, respectively) reduces the degradation of EGFP-SV2 in the DKD background.

2) Fig. 6. The authors used lysosomal, proteasomal and E1 inhibitors to show that the protein decrease in the knockout condition is due to ubiquitination and targeting to the proteasome and lysosome. Indeed they observe a significant increase in the levels of proteins in the presence of these inhibitors when piccolo and bassoon were knocked down. However, no accumulation of proteins was observed in the control condition. Does this mean that degradation of presynaptic proteins is normally not affected by these pathways? Also, this finding is not compatible with the effect of UbK0 in increasing the protein levels also from control (Fig. 7).

These are legitimate concerns, but there is actually a simple explanation for the observed differences in synaptic protein accumulation between Figures 6 and 7. In Figure 6, neurons were treated with the various pharmacological inhibitors for only 16-18 hours. Since our cycloheximide-chase experiments suggest that the half-lives for presynaptic proteins **in control neurons** are typically >24 hours (see Fig. S3), we would not expect to see significant changes in the levels of EGFP-SV2 or other presynaptic proteins in these neurons within the time period of our drug treatments. In contrast, neurons were infected with knockout ubiquitin at the time of plating (0 DIV), giving this molecule ample time (~14 days) to become incorporated into ubiquitin linkages and to inhibit protein polyubiquitination (and thereby degradation) in both control and DKD neurons. The partial rescue of the DKD phenotype by KO Ub likely reflects incomplete incorporation of this molecule into ubiquitin linkages, and/or protein degradation by other pathways, such as macroautophagy (as suggested below).

3) Fig. 5. The authors observe that the abnormal vesicles that accumulate when piccolo and bassoon are knocked down are positive for lysotracker. They also observe that lysosomal inhibition by chloroquine and leupeptin are the most efficient in preventing protein degradation when piccolo and bassoon are suppressed (Fig. 6). Since lysosomal degradation of proteins may be predominantly linked to non-ubiquitinated proteins, is it possible that the lysosomal degradation in the double knockdown condition may not be related to protein ubiquitination only? Is it possible that macroautophagy may also be involved?

This is a very interesting question, and an area of ongoing investigation in the Garner laboratory. We do have some preliminary evidence that the macro-autophagy pathway is upregulated in DKD neurons, but this data is far from conclusive and beyond the scope of the current manuscript. However, in the discussion section (**p. 20**) we now include comments about a potential role for macroautophagy in mediating protein and synaptic vesicle degradation in DKD neurons.

4) Fig. 7D. The data on the ubiquitination of presynaptic proteins could be improved. For instance, the levels of immunoprecipitated proteins should be shown for both Munc13 and VAMP2. Also, it is not clear if the detection with ubiquitin antibodies reveals polyubiquitination since VAMP2 does not appear as a smear and Munc13 had the region of high molecular weight cut. Fig. 7E should also be done again since the current one probably reflects one or very few attachments of ubiquitin rather than polyubiquitination, which should be driving the degradation of proteins based on Fig. 7A using UbK0. If the polyubiquitination is robust in the absence of piccolo and bassoon, perhaps the addition of epoxomicin with or without lysosomal inhibitors may facilitate observing the differences in ubiquitination.

We thank this reviewer and reviewer 3 for bringing this important point to our attention. We agree that polyubiquitinated VAMP2 and Munc13 should exhibit size shifts of >30kD, and we have repeated these experiments to look for higher molecular weight bands of these proteins that are immunoreactive for ubiquitin. Because of the difficulties associated with detecting Munc13-1 and other high molecular weight proteins >250 kD (i.e. inefficient loading/transfer during SDS-PAGE),

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we focused on VAMP2 and synaptophysin. These synaptic vesicle-associated proteins are small (~19 and 38 kD, respectively), abundant, and readily immunoprecipitated from synaptosomes prepared from SC and DKD-expressing neurons (following 8 hr treatment with epoxomicin and lysosome inhibitors, as suggested by the reviewer). As shown in our revised **Figure 7D and E**, we do indeed see that higher molecular weight bands present in VAMP2 and synaptophysin immunoprecipitates exhibit increased ubiquitin immunoreactivity in DKD vs. SC-expressing neurons. To determine whether these bands actually represented ubiquitinated VAMP2 or synaptophysin, we performed an additional experiment. Specifically, we again immunoprecipitated VAMP2 and synaptophysin from synaptosomes, this time prepared from wild-type neurons +/-8 hr treatment with lysosome and proteasome inhibitors. We subsequently immunoblotted this material with a different set of VAMP2 and synaptophysin antibodies. In the inhibitor-treated neurons *only*, we detected VAMP2 and synaptophysin immunoreactive bands at nearly identical sizes to the ubiquitin-positive bands seen in immunoprecipitates from SC and DKD neurons (Figure 7D and E). These findings indicate that the bands represent ubiquitinated forms of VAMP2 and synaptophysin. Moreover, we consistently detect more ubiquitin immunoreactivity in synaptosomes isolated from DKD vs. SC neurons (as seen in the two left lanes of Fig.7D), providing further evidence that synaptic protein ubiquitination is increased in DKD neurons. Together with the ziram and KO Ub data (Figures 6 and 7, respectively), these data strongly suggest that polyubiquitination is enhanced in Bassoon/Piccolo-deficient presynaptic boutons.

5) Fig. 8. The authors mention that siah-1 was pooled out as an interactor of bassoon in a yeast-two hybrid screen. However, no details about the siah-1 region that was isolated in the screenings were provided. Since most siah substrates interact with the region C-terminus to the RING finger (sina domain), the finding that bassoon interacts with the RING finger is particularly interesting. Thus, the siah-1 yeast two-hybrid clone(s) obtained in the screening should also enclose the RING domain, which would confirm the IP data using the fragments of the protein (Fig. 8D). It is also important to mention in Figure S4A the identity of siah-1 construct used to co-transform yeast with the different parts of bassoon.

We have now included this information in the manuscript (**Supplemental Material**). The originally-isolated cDNA clone of rat Siah1 (NM.080905) covered a sequence starting at amino acid 59. The RING domain of Siah1 spans amino acids 39-76, and was therefore not fully covered by the clone isolated. However, this data is consistent with our immunoprecipitation experiments showing that while Bassoon zinc finger 1 preferentially interacts with the RING domain versus the Sina domain, it is most efficiently co-immunoprecipitated with full-length Siah1. These findings indicate that the Bassoon and Piccolo zinc finger domains interact with regions within both the RING and Sina domains of Siah1.

6) Even though more artificial that primary neuronal cultures, I believe that simple biochemistry with transfected HEK293 cells could strengthen the idea that knockdown of piccolo and bassoon activates the catalytic activity of siah-1 leading to increased ubiquitination and degradation of substrates.

This is an interesting idea, but would be technically infeasible. HEK293 cells do not contain Bassoon and Piccolo, and in order to perform the suggested experiment, these molecules would have to be co-transfected into the HEK cells together with three other plasmids (HA-ubiquitin, Siah1, and a Siah1 substrate such as synaptophysin), and subsequently knocked down with an additional vector containing the shRNAs. Since Bassoon contains >10.5 kb of coding region and Piccolo >13 kb (excluding vector backbone), they do not transfect efficiently. In fact, we typically see fewer than 10% of HEK cells expressing these large molecules after transfection, and this is when they are transfected alone. Thus, although it is a nice idea, the proposed experiment is technically very difficult and unlikely to provide useful data about the ability of Bassoon and Piccolo to activate Siah1.

Minor points:

1) The authors mention at the Results section (p. 11) that ubiquitination of one or more ubiquitin molecules target proteins for lysosomal or proteasomal degradation. Although technically correct, this information is not very precise since most proteins targeted to the proteasome require the attachment of four or more ubiquitin molecules. As for the lysosomes, even though multi-

monoubiquitination or K63 polyubiquitination can promote lysosomal degradation of some proteins, it is not clear if proteins in general require ubiquitination to be degraded by this pathway. Therefore, the authors should try to rephrase their explanation.

We have rephrased this sentence as requested (**p. 11**).

2) No details about the yeast two-hybrid system were provided at the Materials and Methods section. This information should be added to the section.

We apologize for this oversight and have now added this information to the Materials and Methods section.

Referee #2

This referee is also positive about the paper, stating "this is an interesting study and the majority of the data are of high quality". The referee thought that a few important points needed to be addressed before publication. We thank the referee for these comments and address his/her concerns below.

1. The data regarding CHMP2 accumulation in neuronal cell bodies are less than compelling. Whether or not these antibody stainings are specific seems doubtful based on the hazy images provided. I'd rather suggest to go for other MVB markers or alternatively to make sure that the signal is specific (i.e. by comparison with KD cells). Also, does one observe transport intermediates in axons in DKD neurons that undergo retrograde traffic? If so, do these stain positive for MVB markers? This should become particularly overt if lysosomal degradation is blocked by inhibitors.

We would like to note that immunostaining of rat brain tissue with this CHMP2b rabbit polyclonal antibody from Abcam (ab33174) has been previously published (Belly et al., 2010), and we have confirmed its specificity by Western blot (see **Response Figure 1** below). Nevertheless, we have included new images of CHMP2b immunostaining in cell bodies and axons for **Figures 5 and 9**. In all of our experiments (performed in two different laboratories and on four independent batches of neurons), we find that CHMP2b fluorescence intensity is significantly increased in the cell bodies of DKD vs. SC neurons. In response to the reviewer's questions, we have now also investigated whether CHMP2b immunoreactivity is increased in DKD-expressing axons. Our data reveal that CHMP2b fluorescence intensity is similarly elevated (by nearly threefold) in DKD-expressing axons, further supporting the concept that MVBs are upregulated in response to Bassoon/Piccolo knockdown.

With regard to the reviewer's query of whether axonal retrograde transport of MVBs can be detected in DKD neurons, we have repeatedly tried to perform live imaging of mCherry-CHMP2b expressed in DKD neurons. Unfortunately, we find that overexpression of this protein, as well as other proteins found in degradative pathways (LAMP2 and wild-type ubiquitin), causes cellular toxicity and death of DKD neurons within 2-3 days (vs. limited or no toxicity in SC neurons). These observations are consistent with the concept that multiple degradative pathways are upregulated in DKD neurons, and that any additional "degradative load" is not tolerated.

2. I am puzzled by the fact that the claimed ubiquitinated form(s) of VAMP2 in Fig. 7 runs at about 18 kDa, i.e. the MW of VAMP2 in the ABSENCE of Ub! This is odd and makes me wonder about the quantifications. Clearly, better and more conclusive evidence in favor of the accumulation or formation of Ub-conjugated presynaptic proteins is required. This is a key point in my opinion.

This concern was shared by Reviewer 1 (see point #4 and our rebuttal above), and we agree that we did not adequately present or analyze this data in the previous version of the manuscript. We now include new data in **Figures 7D and E**, demonstrating that both VAMP2 and synaptophysin exhibit increased polyubiquitination in synaptosomes isolated from DKD-expressing neurons. Together with our ziram and KO Ub experiments (Figures 6-7), these findings strongly suggest that polyubiquitination of presynaptic proteins is enhanced in DKD neurons.

3. There is a poor match between the quantifications in Fig 1C and the blots shown in panel B. In fact, in none of the lanes do I see any evidence of presynaptic proteins loss at DIV9, although the quantifications in panel C suggest a profound loss of at least Picc and Bsn at this stage. The same

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concern holds true for the images in Fig. 2B: The DKD neurons shown appear to contain plenty of Picc and no difference to the scrambled control is overt.

In our opinion, the blots shown in the original Figure 1B depicted clear decreases in Bassoon and Piccolo levels in DKD-expressing neurons at 9 DIV (though we acknowledge that knockdown is less complete at 9 DIV than at 14 DIV). Nevertheless, we have replaced the 9 DIV Bsn and Pclo panels with different images that are perhaps more equivalent to the average values shown in Figure 1C. In addition, we have indicated the most prominent isoforms of Piccolo with arrows. Note that the lower molecular weight isoform (<460 kD) is still present at 9 DIV but absent by14 DIV, indicating that it takes longer to knockdown.

With respect to the comments about Figure 2, we would like to clarify what is being depicted in this figure, since it appears to be a source of confusion for both reviewers 2 and 3. Our lentiviral vector coexpresses the Bsn and Pclo (or scrambled control) shRNAs together with an EGFP-tagged reporter protein, in this case EGFP-SV2 to label presynaptic boutons. Although we routinely infect nearly ~100% of neurons/flask or coverslip for certain experiments (such as the immunoblotting in Figure 1), we typically infect only 20-30% of cells/coverslip for immunofluorescence experiments. Thus, in panels A and B of Figure 2, ***only the axons expressing EGFP-SV2 contain the SC or DKD shRNAs***. In DKD-expressing neurons, EGFP-SV2 puncta (representing presynaptic boutons; white arrows) clearly lack Bassoon and Piccolo immunoreactivity and therefore exhibit very low levels of colocalization with these proteins (quantified in Figure 2, panels C and D). In contrast, EGFP-SV2 puncta in SC-expressing neurons exhibit a high degree of colocalization with Bassoon and Piccolo immunostaining, indicating that normal levels of these proteins are present within presynaptic boutons of control neurons. Since only 20-30% of neurons are infected for each condition, the majority of Bassoon and Piccolo immunostaining present in panels A and B of Figure 2 comes from wild-type, uninfected neurons. We have tried to clarify our description of these experiments in the Results section (**p. 7-8**), in order to prevent further confusion about this issue.

4. I miss any kind of evidence that the ability of Siah1 to foster presynaptic degradation is related to its ability to bind to Bsn/ Picc. For example, one would predict that OE (see S5E, F) of a Bsn/ Picc binding defective mutant of Siah1 should have no effect on presynaptic protein levels similar to an enzymatically inactive version. This should be straightforward to test.

This is a very nice idea, but it has not been straightforward to test. Siah1 and other RING domain E3 ligases serve as adaptor proteins that bring together E2 ubiquitin conjugating enzymes (which bind to their RING domains) and protein substrates (which bind to another domain of the ligase – in the case of Siah1, the Sina domain). Thus, any mutation that disrupts either the RING or Sina domain will inactivate Siah1. Our initial biochemical and molecular mapping studies reveal that the Bsn/Pclo zinc fingers interact preferentially with Siah1's RING domain, but that this interaction is stabilized through the Sina domain. Thus, we are facing extensive structure/function studies to identify specific residues on Siah1 that impair binding to Bsn/Pclo, but do not interfere with its activity. Such experiments are beyond the scope of this already long and data-rich manuscript.

5. As rescues by Siah1 KD or OE of the Zn finger domains of Picc/ Bsn are incomplete I would suggest to discuss possible explanations for this; i.e. other degradation pathways or E3 ligases that may associate with the AZ.

We have now discussed this issue on **p. 20** in the Discussion section.

Referee #3:

This referee was also very positive about the manuscript. He/she wrote, "I found the manuscript very interesting and overall convincing. The data set is extensive… The experiments are very well conceived and carried out…The manuscript is well written and very clear." He/she also stated, "These experiments conclusively establish the mechanistic cause of the degradation seen in the absence of Bassoon and Piccolo. These data will be extremely interesting to anyone studying synaptogenesis and synaptic remodelling." We thank the referee for these very enthusiastic comments, and address his/her minor concerns below.

1. I find the lack of consistency between the Western Blots of Fig. 1B-C and the stainings in

f.instance Fig. 2 confusing. It seems that at DIV14, most tested proteins are basically gone from the Western (Fig. 1B), but in Fig. 2B, the stainings for Piccolo and Bassoon at DIV14 look normal. Why is this? The fluorescence in the immunostaining should be quantified and compared between knockdown and control construct, and the degree of reduction compared to the Western. In addition, the experiments in Fig. 2 (and most of the rest of the paper) were carried out with another viral construct (expressing EGFP-SV2 instead of EGFP) than the construct used for protein quantification in Fig. 1. Given the discrepancy between Fig. 1 and 2, the authors should make sure that there is no difference in the degree of knockdown between constructs (for instance because of a lower virus titer of the longer construct), and this should be mentioned in the paper.

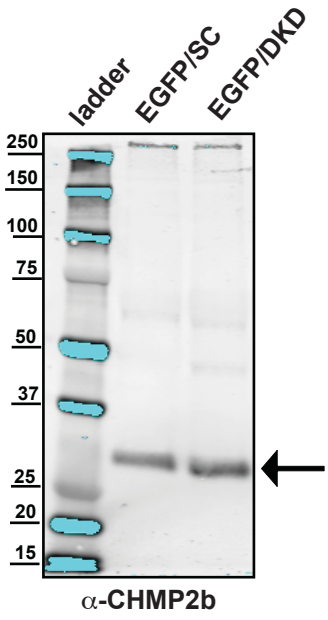
Please see our response to point #3 raised by Reviewer 2, who had a very similar concern. It seems that we were not clear enough in our explanation of how we performed lentiviral infections in order to obtain data for Figures 1 and 2. To reiterate: the immunoblots shown in Figure 1B were performed with lysates prepared from neurons infected with extremely high titer lentivirus. Therefore, nearly 100% of neurons expressed the SC or DKD shRNAs, leading to almost-complete knockdown of Bassoon and Piccolo in a large number of neurons. In contrast, the images shown in Figure 2A and B were obtained from neuronal cultures infected with lower titer virus, such that only 20-30% of neurons expressed the EGFP-SV2/SC or EGFP-SV2/DKD constructs. Bassoon and Piccolo immunostaining are absent ***only*** within DKD-expressing presynaptic boutons, which are labeled with EGFP-SV2. Thus, all Bassoon and Piccolo immunostaining in the DKD panels comes from presynaptic boutons of wild-type, uninfected neurons.

2. The lack of colocalization between EGFP-SV2 and piccolo/bassoon. I am not convinced that this is a real phenomenon, or what it would mean if it were. It might be a consequence of the changes in intensity of SV2 and/or Piccolo/Basson spots, which makes it harder to detect the colocalization. If the phenomenon is real, I guess it might mean that those synapses completely devoid of Piccolo/Bassoon experience an even larger breakdown of EGFP-SV2? Or what is the author's interpretation of these results? - formation of SV2-clusters outside of synapses?

Again, please see our comments for the previous point. The lack of colocalization between EGFP-SV2 and Piccolo/Bassoon in DKD neurons demonstrates effective knockdown of these proteins, leading to their loss from presynaptic boutons. The decreased intensity of EGFP-SV2 in DKD boutons reflects its rapid degradation in the absence of Bassoon/Piccolo, a fate shared by multiple presynaptic proteins and demonstrated by our experiments throughout the manuscript.

Reference

Belly A *et al.* CHMP2B mutants linked to frontotemporal dementia impair maturation of dendritic spines. *J Cell Sci* 123:2943-54 (2010).



Response Figure 1: Immunoblot of lysates from SC or DKD-expressing hippocampal neurons, probed with CHMP2b rabbit polyclonal antibody (Abcam, catalog #ab33174). Note the very specific labeling of this antibody at ~ 32 kD (arrow), the predicted size for CHMP2b.

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Thank you for submitting your revised manuscript to the EMBO Journal. The manuscript has now been re-reviewed by the referees. As you can see below, the referees appreciate the introduced changes and support publication here and find the paper interesting and important. I am therefore very pleased to accept the paper for publication here.

We also now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

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Thank you for contributing to the EMBO Journal!

Referee #1

The authors of the manuscript "Bassoon and Piccolo maintain synapse integrity by regulating protein ubiquitination and degradation" addressed all major points raised by the reviewer. They added several new experiments and answered in a very meticulous way to the questions. They now showed unequivocally that knockdown of piccolo and bassoon lead to increased ubiquitination and degradation of synaptic proteins.

The article is original and shows in a very convincing manner that the ubiquitin-ligase SIAH is important to control the protein homeostasis at the synapse. This study is exciting not only to the field of synapse formation but also for those that study SIAH in different contexts. The notion that different proteins can tightly control SIAH ubiquitin-ligase activity seems unique among the ubiquitin-ligases. Therefore, I strongly recommend this study to be published at the EMBO J as it is.

Referee #2

All my comments have been adequately addressed and I thus recommend publication of this excellent Ms in The EMBO J.

Referee #3

The authors have answered my few concerns and clarified these issues in the paper. The manuscript is very interesting and important, and it should be published in EMBO J.

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